

Methodologies for EGFR Mutation Detection

AstraZeneca has compiled this document and believes it provides useful information for laboratories and health care professionals. Whilst AstraZeneca has taken reasonable care in compiling this document and the information contained in it, we are not responsible for any action taken by any person or organisation, wherever they are based, as a result, direct or otherwise, of information contained in this document. Nothing in this document should be construed as the giving of advice or the making of any recommendation and this document should not be relied upon as the basis for any decision or action. Nothing in this document should be construed as granting any right or licence to use intellectual property owned by us or any third party.

Overview

- What is EGFR Mutation Testing?
- Which mutations are important in the context of IRESSA™ (gefitinib)?
- Which samples are utilised for EGFR Mutation Analysis?
- What tools are available for EGFR Mutation Testing?
 - Description of the most commonly used methods: PCR/Sequencing and ARMS
 - Less commonly used methods
- Example of how to report EGFR Mutation Results

What is EGFR Mutation Testing?

EGFR Mutation Testing

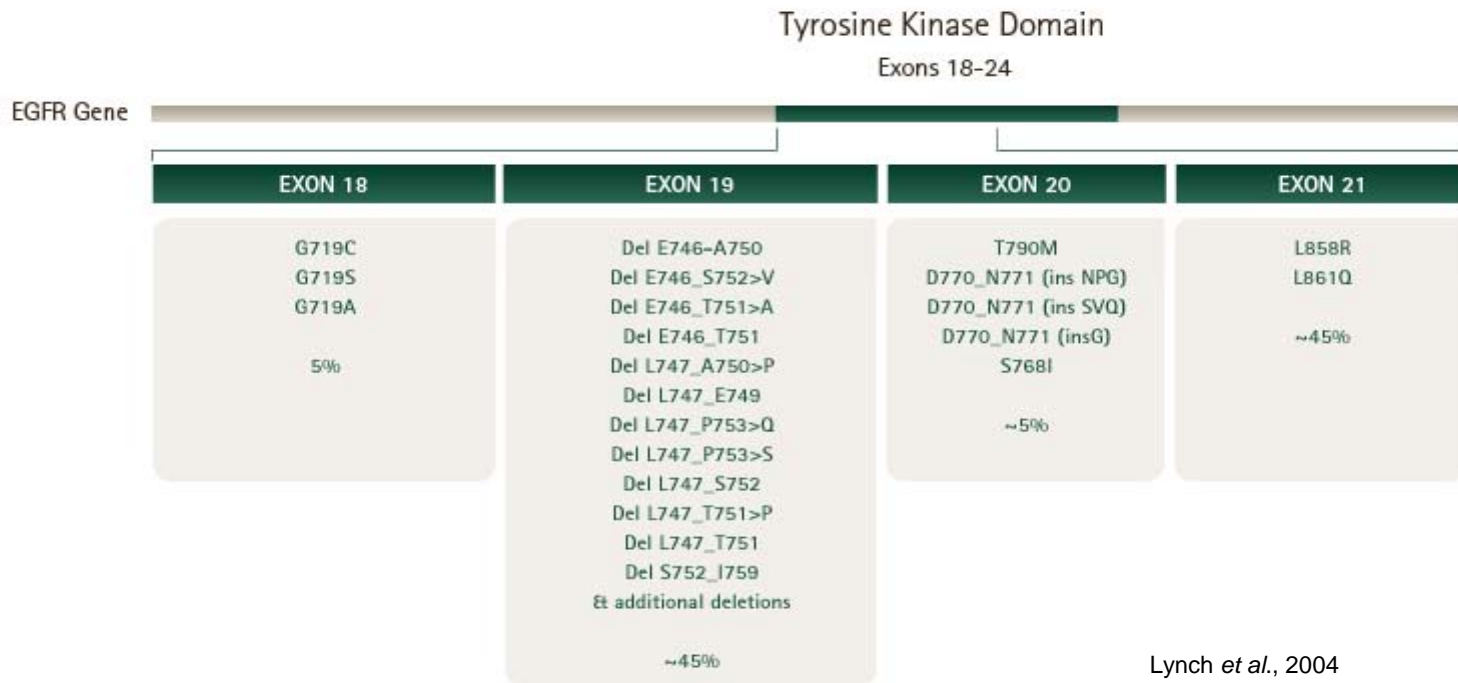
- EGFR mutation testing involves the analysis of mutations in tumour DNA encoding the EGFR gene
- **It is not**
 - the measurement of the number of copies of DNA of the EGFR gene (analysed by Fluorescent In-Situ Hybridisation, FISH)
 - the measurement of the expression of EGFR protein in the cell (analysed by Immunohistochemistry, IHC)

EGFR MEASURE	TEST MATERIAL	LOCATION OF TEST MATERIAL	METHOD OF DETECTION
EGFR Mutation	DNA	Nucleus	Mutation Analysis e.g. Sequencing, ARMS, other
EGFR Copy Number	DNA	Nucleus	Fluorescent In-Situ Hybridisation (FISH)
EGFR Expression	Protein	Cell Membrane	Immunohistochemistry (IHC)
EGFR RNA Expression	RNA	Nucleus	RT-PCR

What Mutations Are Important in the Context of IRESSA™ (gefitinib)?

Spectrum of EGFR Mutations

- EGFR mutations are found in four exons of the EGFR gene, exons 18 to 21
- Exon 19 deletions and exon 21 L858R mutations account for ~90% of all mutations
- Screening technologies such as sequencing normally assess all four exons
- Targeted technologies tend to assess specific common mutations



Lynch *et al.*, 2004
 Paez *et al.*, 2004
 Sharma *et al.*, 2007
 Hirsch and Bunn, 2009

Recommendation on which EGFR Mutations should be tested in relation to use of gefitinib in NSCLC (1)

- Tumour cells harbouring EGFR mutations become "oncogene (EGFR) addicted" and undergo an apoptosis in response to gefitinib exposure which translates in the clinic into a significant tumour shrinkage in the majority of the patients with such tumours
- The science and clinical data in the area of EGFR mutations are still emerging
- Based on previous experience from randomised phase III studies (IPASS, INTEREST, V-15-32, ISEL) and external literature from before March 2009, **exons 18-21** of the EGFR gene should be analysed to support the treatment decision for a patient with advanced NSCLC

Recommendation on which EGFR Mutations should be tested in relation to use of gefitinib in NSCLC (2)

Category	EGFR mutation description	% of known activating EGFR mutations	Data supporting sensitivity to gefitinib	
1	Exon 19 deletions; L858R	~90%	YES	Exon 19 deletions and the L858R mutation constitute ~90% of the EGFR mutations identified to date. In patients with tumours that are positive for these mutations, the current data supports sensitivity to gefitinib
2	T790M/exon 19 deletions; T790M/L858R; G719X; L861Q; S768I	~7%	LIMITED[#]	NSCLC patients can have tumours that are positive for more than one EGFR mutation type. These are known as double mutations and are predominantly seen with T790M & an exon 19 deletion or T790M & L858R. In patients with tumours positive for these mutations where T790M is present, or tumours with other rare mutations listed here, there is very limited data to support sensitivity or resistance to gefitinib
3	T790M alone; exon 20 insertions; other mutations	~3%	NONE[#]	The exon 20 point mutation T790M (T790M alone), and the exon 20 insertions make up ~3% of the EGFR TK mutations identified to date. In patients with tumours positive for these mutations, there are currently no data to support sensitivity to gefitinib. Some screening methodologies such as sequencing may identify novel EGFR mutations where there will be no clinical or pre-clinical data to guide use of gefitinib

[#] Due to lack of data it is difficult to draw definitive conclusions on the sensitivity or lack of sensitivity to gefitinib in these mutation types, because they only constitute ~10% of all EGFR TK mutations. Few patients in global AstraZeneca studies have been identified with these types of EGFR TK mutations

Mok *et al.*, 2008
 Kim *et al.*, 2008
 Hirsch *et al.*, 2006
 AZ In-House Data - Unpublished

Factors Enriching for Mutation Status

Summary of multivariate logistic regression analysis to identify factors that independently predicted for the presence of EGFR mutations in 786 Caucasian patients*

Factors that predicted for presence of EGFR mutation	p-value	Odds of EGFR mutation	Positive predictive value (9.5% of the overall population are EGFR mutation-positive (M+))
Smoking status	<0.0001	6.5 times higher in never smokers than ever-smokers	28/70 (40%) of never smokers are M+ 47/716 (7%) of ever smokers are M+
Histology	<0.0001	4.4 times higher in adenocarcinoma than in non-adenocarcinoma	63/396 (16%) of patients with adenocarcinoma histology are M+ 12/390 (3%) of patients with non-adenocarcinoma histology are M+
Gender	0.0397	1.7 times higher in females than males	40/235 (17%) of females are M+ 35/551 (6%) of males are M+

*from the following studies: INTEREST, ISEL, INTACT 1&2, IDEAL 1&2, INVITE

Samples Used for Mutation Analysis

Importance of Samples Enabling Mutation Analysis

- At diagnosis, a sample undergoes histological evaluation
- Practices differ on regions and may involve
 - Tumour biopsy (usually Formalin Fixed Paraffin Embedded)
 - Cytology
 - Other
- Tumour biopsy is the most common and preferred sample – referred to as the “gold standard” for mutation analysis
- Surrogate tissues such as plasma and serum are being evaluated
- **Recommendations**
 - Where possible, collect a biopsy for histological evaluation and mutation analysis
 - Allow sufficient material (5-10 5 micron sections) for DNA extraction and mutation analysis, preceded by macrodissection if possible
- Key message: without a suitable sample, mutation analysis is impossible.

Samples Used for EGFR Mutation Detection

A. Tumour Samples

- Routine practice and so-called “gold-standard” is to analyse DNA derived from a tumour sample, normally Formalin Fixed Paraffin Embedded (FFPET) diagnostic block.
- DNA tends to be degraded, therefore, it is important to use robust well validated extraction methodologies to avoid assay fails and false negative results where possible.
- Frozen tumour yields higher quality DNA but is rarely available

B. Surrogate Tissues

- Due to difficulty in obtaining biopsy samples and lack of tumour sample in some cases, the use of surrogate samples (non-tumour) is increasing
- These include:
 - Serum
 - Plasma
 - Cytology (Induced Sputum, other)
 - Bronchial Alveolar Lavage or Bronchial Scrapings
- Adoption of testing on surrogate tissues requires further work prior to use in routine clinical/diagnostic practice

The Future of Mutation Analysis

- Some of the existing methods for identifying EGFR mutations are validated only on histological material
- There are attempts to develop assays for other more readily available surrogate samples such as plasma, serum, cytology and pleural fluid which may contain tumour DNA. All of these surrogates are of great interest for mutation analysis
- In particular, blood based (serum/plasma) testing is particularly attractive due to the ease of accessing such samples. However, the sensitivity of the methods currently used mean that identifying EGFR mutation positive patients from blood is still challenging
- AstraZeneca research suggests that of the patients who are identified as mutation positive by analysing their tumour, approximately 50% can be identified as mutation positive by analysing circulating free DNA (cfDNA) in blood. Data demonstrated a 0% false positive rate.
- For this reason, blood based testing may not replace biopsy-based testing in the near future. However, it could be used in addition to biopsy testing, for those patients for whom the physician does not have access to a sample
- Technology is constantly developing. Further research in this setting may alter mutation testing practices in the future, particularly in those patients without biopsy samples available for mutation analysis

Preparation of DNA for Mutation Analysis

- Extraction of sufficient yields of sufficient quality DNA is key to successful mutation analysis
- Many methods are available, including efficient spin column methods
- Pathology review of the tissue is required prior to analysis to ensure presence of tumour cells
- Macrodissection or scraping of the tumour area from the slide to the extraction will decrease normal contaminating cells and risk of false negative results.
- DNA quantification is a useful step prior to mutation analysis
 - Quantitative-PCR (Q-PCR) is a useful tool to provide information about the quantity and quality of the DNA sample.
 - Q-PCR measures amplifiable DNA in a sample, taking into account the fact that the DNA from archival tumour samples tends to be fragmented and a proportion of the sample may not be amplified.
 - This is superior to a spectrophotometric method

What tools are available for EGFR Mutation Testing?

Detection of EGFR Mutations

- There is a wide choice of methods available for mutation detection
- Many have been adapted from genotyping technologies of non-tumour DNA
- Many of the technologies available for Kras testing are also available for EGFR mutation analysis, and vice versa
- The technologies can be divided into two subgroups, screening and targeted mutation detection technologies

METHOD	Mutation Screening Technology: Samples are screened for <u>all</u> EGFR mutations, known and novel variants	Targeted Mutation Detection: Samples are analysed for <u>known</u> EGFR mutations only
PCR/Sequencing	✓	
Nested PCR/Sequencing	✓	
PCR/HRMA/dHPLC (Melt Analysis)	✓	
Pyrosequencing	✓	
ARMS		✓
PNA/LNA Clamp		✓
SNAPSHOT		✓
PCR/Fluorescent RFLP		✓
ME PCR/ Sequencing		✓
PCR Invader		✓

Screening vs. Targeted Technologies

Briefly, the relative merits of the two approaches are:

a) Screening technologies for mutation detection (E.g. DNA sequencing)

- Advantages:
 - All variants, including novel variants may be detected
 - Platform is available in many molecular genetics laboratories
- Disadvantages:
 - Sensitivity of the technology tends to be lower than targeted methods (10-30% mutant DNA in normal DNA background of heterogeneous tumours)
 - Requires experienced operators
 - Tends to be more labour intensive

b) Targeted mutation detection (E.g. ARMS)

- Advantages:
 - Only mutations assayed may be detected therefore less time consuming
 - Sensitivity is increased compared to screening technologies (1% mutant DNA in normal DNA background of heterogeneous tumours)
- Disadvantages:
 - Mutations not assayed for may be missed
 - Depending on source, reagents may be more expensive

Choosing a Method for EGFR Mutation Analysis

- The choice of method is influenced by a number of factors including
 - Availability of instrumentation
 - Availability of reagents required
 - Amount of tumour material available for testing
 - Lab preferences and practices
 - Regional preferences
 - Cost and reimbursement
- Currently, the most common methods available for EGFR mutation detection are PCR/Sequencing and ARMS analysis
- Increasingly, other methods are being adopted as technology evolves

Common Testing Practices: PCR/Sequencing and ARMS

EGFR Mutation Kit – ARMS based

- Currently, the only kit available for EGFR mutation analysis is the TheraScreen EGFR29 Mutation Test Kit from DxS.
 - CE marked IVD
 - Link to DxS Website
- This kit is an ARMS based method which analyses 29 mutations within the EGFR gene:
 - 19 Deletions in exon 19
 - T790M
 - L858R
 - L861Q
 - G719X (G719S/G719A /G719C)
 - S768I
 - 3 insertions in exon 20
- As of June 2009, the TheraScreen EGFR29 kit has been CE-marked and validated for use on the ABI 7500 real-time PCR instrument. Validation on the Roche Lightcycler (www.roche.com) is planned, and may be available at the start of 2010.
- Other instruments may require additional validation

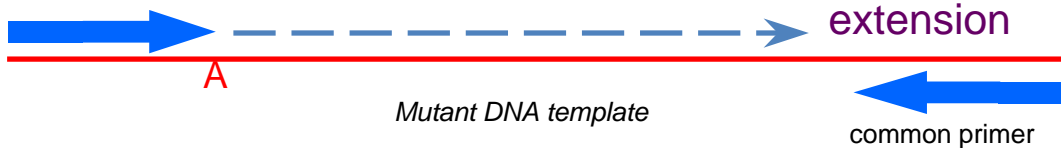
Newton *et al.*, 1989

Whitcombe *et al.*, 1999

ARMS for EGFR Mutation Analysis: How does it work?

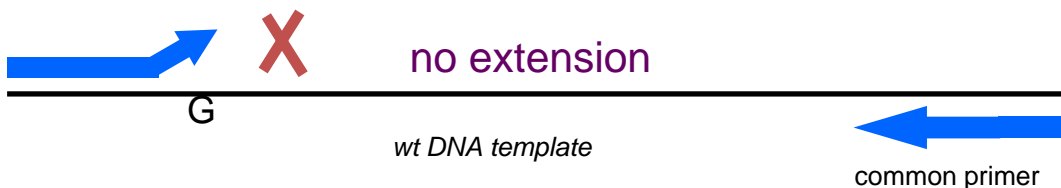
- ARMS: Amplification Refractory Mutation System (& TaqMan or Scorpions)
- Highly sensitive method for mutation detection (<1% sensitivity)
- Allows detection of targeted mutations only
- Small assay (100-150bp) often leads to increased success, particularly when analysing DNA from Formalin-Fixed Paraffin Embedded Material
- Kit based method is accompanied by analysis and quality guidelines, allowing for standardisation across laboratories and regions
- Samples deemed mutation positive, but close to the stipulated cut-off should be repeated for verification

mt ARMS Primer



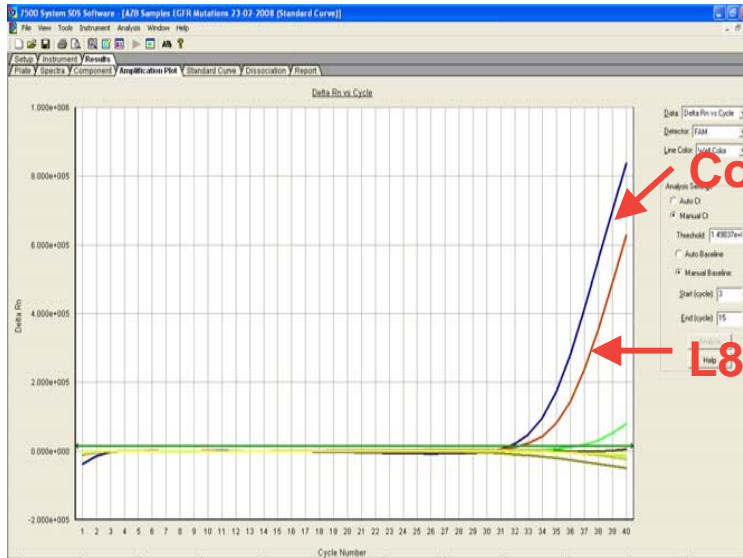
Mutation present
= Extension
= Signal

mt ARMS Primer

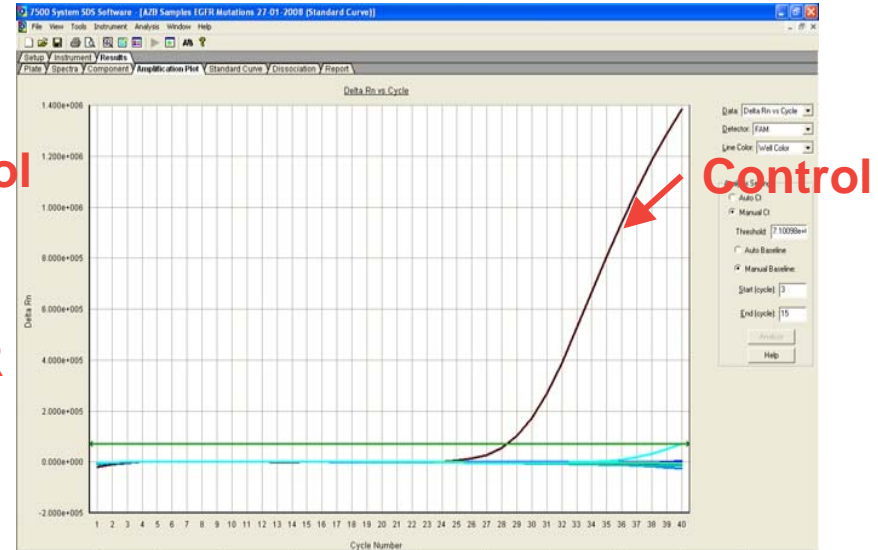


Mutation absent
= No Extension
= No Signal

Example of ARMS data



Mutation Positive



Mutation Negative

- A real-time PCR instrument is required to perform PCR amplification and to measure fluorescence (ABI 7500/7700, MX3000, Roche LightCycler are amongst the available RT-PCR Instruments)
- ~ 2 hours/run

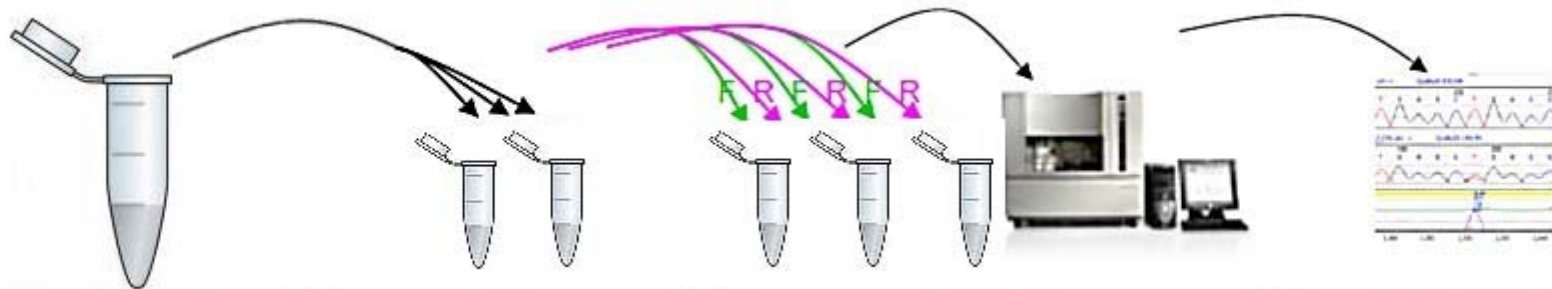
Non-Kit Based Methods for EGFR Mutation Detection

- Many non-kit based methods are available
- In this case, laboratories source their own reagents
- PCR/Sequencing is the most common method utilised and involves screening exons 18, 19, 20 and 21
- As a screening technology, sequencing has the ability to detect both known and novel mutations in the EGFR gene.
- If a novel mutation is detected, it is important to
 - Verify its presence in an independent reaction
 - Determine whether it is a tumour variant, or a rare host polymorphism

PCR/Sequencing for EGFR Mutation Analysis: Key Points

- Requires PCR amplification of DNA, fluorescent dye-incorporation for DNA sequence detection
- Less sensitive method for mutation detection (10-30% sensitivity)
- Allows screening of all mutations present in the sample in exons 18, 19, 20 and 21
- Large assay (150bp – 250bp) may lead to assay fails, particularly when analysing DNA from Formalin-Fixed Paraffin Embedded Material
- Artefacts (false peaks) may be detected. If a novel mutation is detected, it is important to avoid reporting false positive results by
 - Verifying the presence of variants in an independent reaction
 - Determine whether it is a tumour variant, or a rare host polymorphism
- Quality guidelines are not widely available. Individual labs and regions should implement a quality system.
- Experienced users required for sequence analysis

PCR/Sequencing for EGFR Mutation Analysis: Summary of the Process



DNA Extraction

DNA is extracted from a Formalin Fixed Paraffin Embedded (FFPET) sample
E.g. Diagnostic Biopsy

PCR

The sample is amplified in 3 separate reactions if possible. Independent PCRs increase confidence that a mutation is real (true positives) rather than artefactual (false positives)

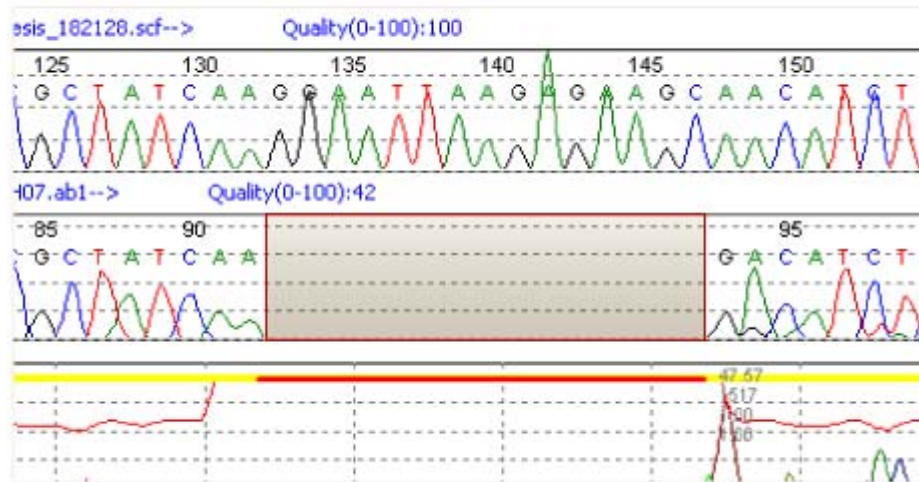
Sequencing

Sequencing Reaction of the PCR products is carried out, in both Forward (F) and Reverse (R) directions, in each of the three independent amplifications
The fluorescently labelled products are then run on a genetic analyser or sequencer.

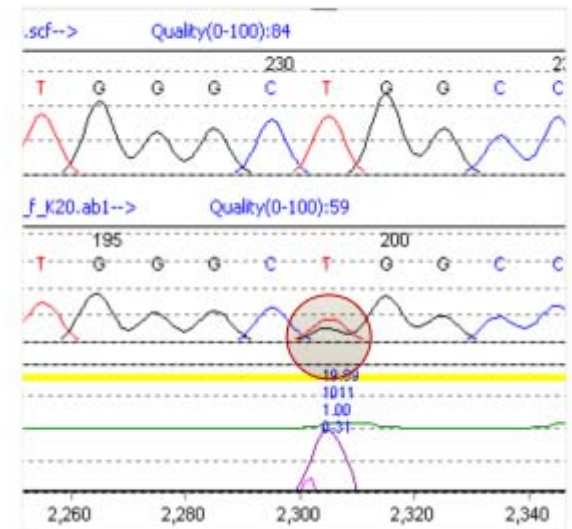
Sequence Analysis

Sequence traces are analysed using suitable sequencing software, where mutation calls (positive vs. negative) are made. Verification of novel mutations or those that occur in only one amplification reaction is required.

Example of Sequencing Data



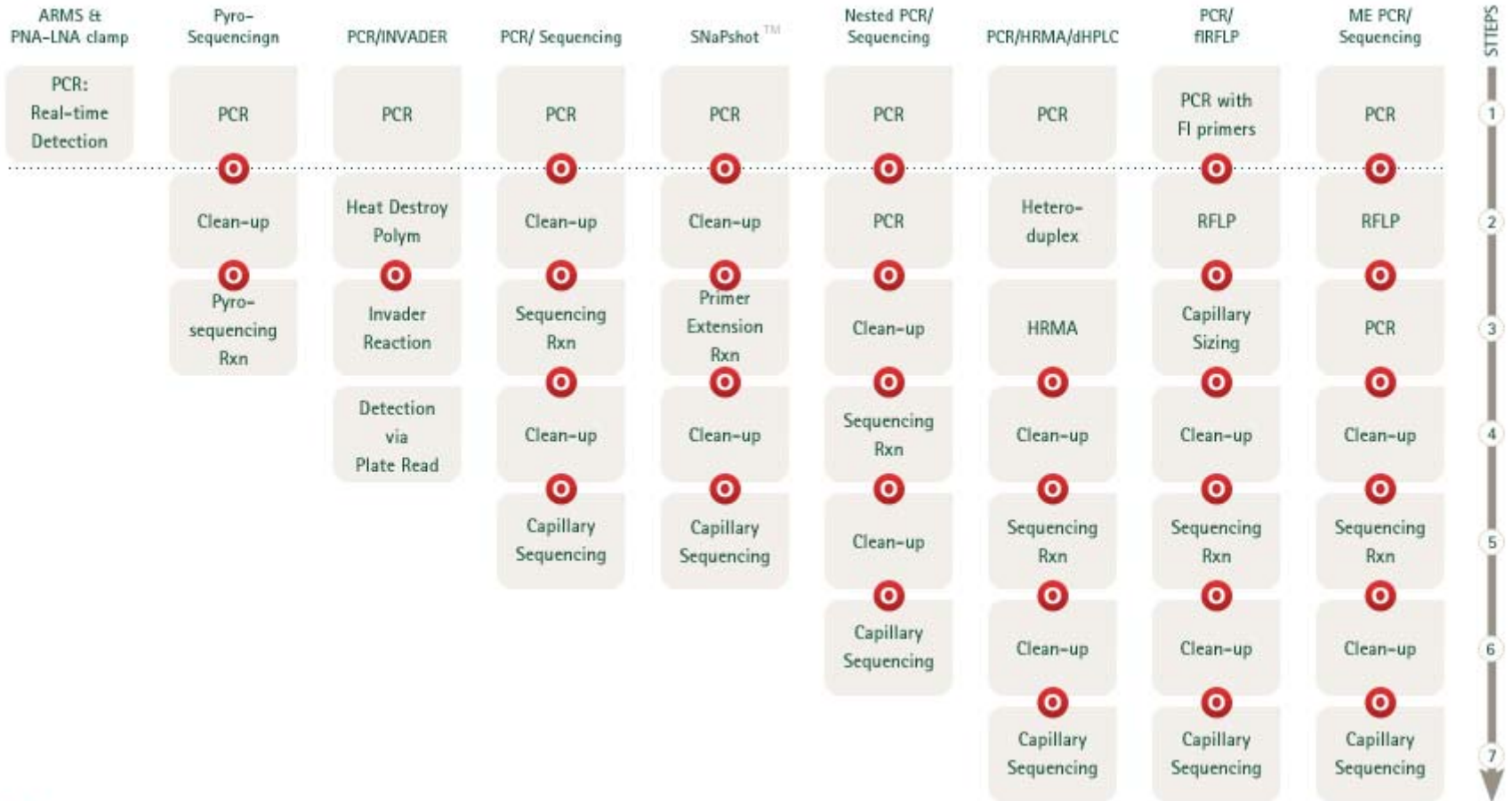
Exon 19 deletion E746-A750



Exon 21 L858R

Other Available Methods

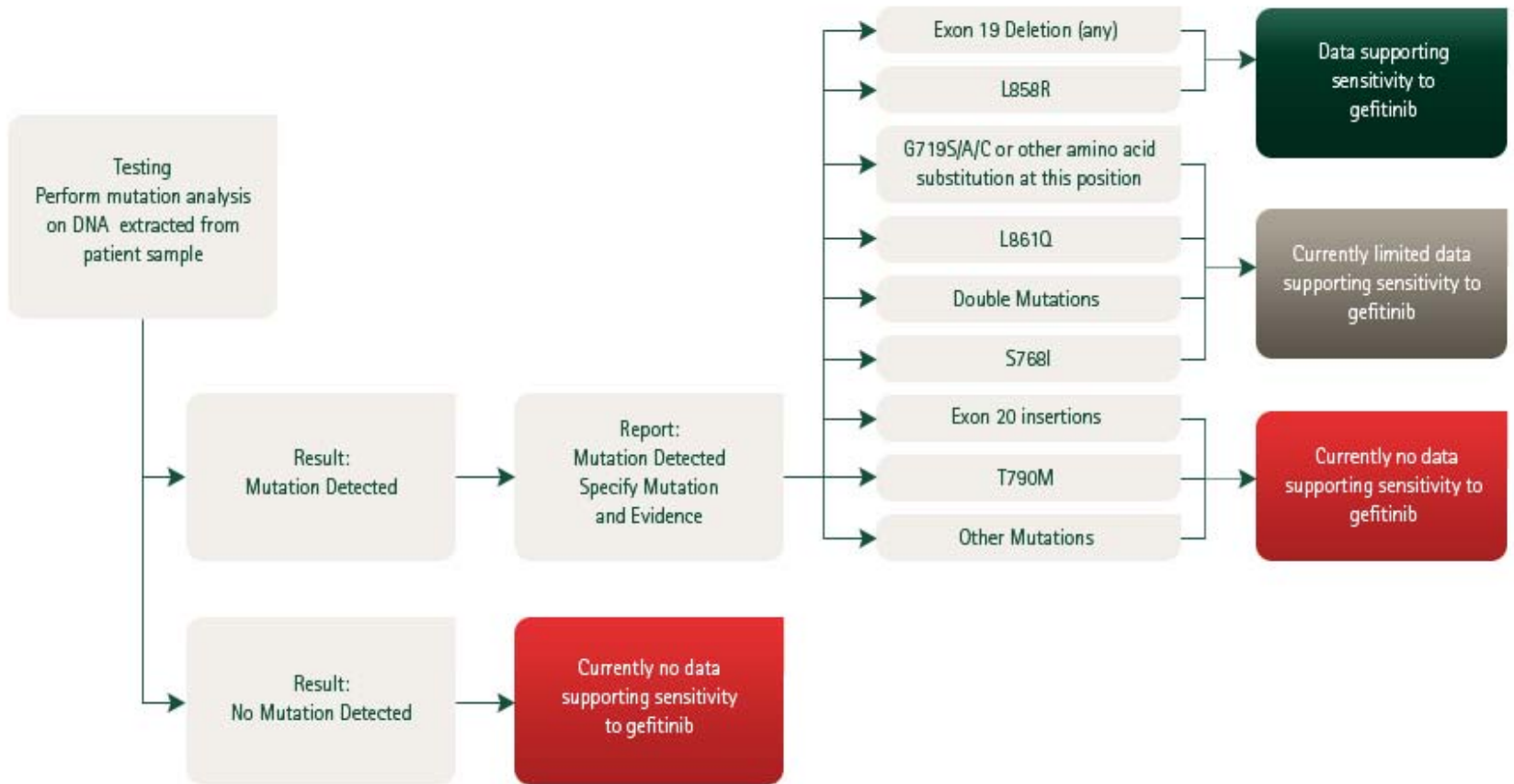
Methodology Steps (Following DNA extraction)



○ = Transfer product /add reagent/ open tube

How to Report EGFR Mutation Results

Decision Tree



EGFR Mutation Result Report Form

EGFR Mutation Test Results

PATIENT & TEST DETAILS

Patient Name:		Lab Accession Number:	
Date of Birth:		Tissue Specimen Site:	
Age:		Date Patient Sampled:	
Sex:		Date Sample Received:	
Referring Physician:		Date Result Reported:	
Record Number:		Requesting Physician:	
Methodology Utilised:	Sequencing/ARMS/Other (Specify)	Comments:	

OVERALL ASSAY RESULT

Mutation Positive	Yes/No
Mutation Negative	Yes/No
Assay Fail	Yes/No

DETAILED ASSAY

Test Name/Assay Name	Mutation Detected?	Recommendation re. gefitinib
Deletions in exon 19	Yes/No/Fail	Data supporting sensitivity to gefitinib
L858R	Yes/No/Fail	
G719S	Yes/No/Fail	Currently limited data supporting sensitivity to gefitinib
G719A	Yes/No/Fail	
G719C	Yes/No/Fail	
L861Q	Yes/No/Fail	
Double Mutations	Yes/No/Fail	
S768I	Yes/No/Fail	
Insertions in exon 20	Yes/No/Fail	Currently no data supporting sensitivity to gefitinib
T790M	Yes/No/Fail	
Other Mutation	Yes/No (Specify Type)	

References

- Lynch TJ, Bell DW, Sordella R, Gurubhagavatula S, Okimoto RA, Brannigan BW, Harris PL, Haserlat SM, Supko JG, Haluska FG, Louis DN, Christiani DC, Settleman J, Haber DA. Activating mutations in the epidermal growth factor receptor underlying responsiveness of non-small-cell lung cancer to gefitinib. *N Engl J Med*. 2004 May 20;350(21):2129-39.
- Paez JG, Jänne PA, Lee JC, Tracy S, Greulich H, Gabriel S, Herman P, Kaye FJ, Lindeman N, Boggon TJ, Naoki K, Sasaki H, Fujii Y, Eck MJ, Sellers WR, Johnson BE, Meyerson M. EGFR mutations in lung cancer: correlation with clinical response to gefitinib therapy. *Science*. 2004 Jun 4;304(5676):1497-500.
- Sharma SV, Bell DW, Settleman J, Haber DA. Epidermal growth factor receptor mutations in lung cancer. *Nat Rev Cancer*. 2007 Mar;7(3):169-81.
- Mok T, Wu, YL, Thongprasert S, Yang CH, Chu D, Saijo N, Jiang H, Watkins C, Armour A, Fukuoka M. Phase III, randomised, open-label, first-line study of gefitinib vs carboplatin / paclitaxel in clinically selected patients with advanced nonsmall- cell lung cancer (IPASS). Oral Presentation. ESMO 2008.
- Kim ES, Hirsh V, Mok T, Socinski MA, Gervais R, Wu YL, Li LY, Watkins CL, Sellers MV, Lowe ES, Sun Y, Liao ML, Osterlind K, Reck M, Armour AA, Shepherd FA, Lippman SM, Douillard JY. Gefitinib versus docetaxel in previously treated non-small-cell lung cancer (INTEREST): a randomised phase III trial. *Lancet*. 2008 Nov 22;372(9652):1809-18.
- Kimura H, Fujiwara Y, Sone T, et al: High sensitivity detection of epidermal growth factor receptor mutations in the pleural effusion of non-small cell lung cancer patients. *Cancer Sci* 97:642-648, 2006

References

- Eberhard DA, Giaccone G, and Johnson BE: Biomarkers of Response to Epidermal Growth Factor Receptor Inhibitors in Non-Small-Cell Lung Cancer Working Group: Standardization for Use in the Clinical Trial Setting. *Journal of Clinical Oncology* 26: (6), 983-993, 2008
- Board RE, Thelwell NJ, Ravetto PF, Little S, Ranson M, Dive C, Hughes A, Whitcombe D. Multiplexed assays for detection of mutations in PIK3CA. *Clin Chem*. 2008 Apr;54(4):757-60.
- Ogino S, Kawasaki T, Brahmandam M, Yan L, Cantor M, Namgyal C, Mino-Kenudson M, Lauwers GY, Loda M, Fuchs CS. Sensitive sequencing method for KRAS mutation detection by Pyrosequencing. *J Mol Diagn*. 2005 Aug;7(3):413-21.
- Newton CR, Graham A, Heptinstall LE et al. Analysis of any point mutation in DNA. The amplification refractory mutation system (ARMS). *Nucleic Acids Res*. 1989; 17: 2503-2516.
- Whitcombe D, Theaker J, Guy SP, Brown T, Little S. Detection of PCR products using self-probing amplicons and fluorescence. *Nat.Biotechnol*. 1999; 17: 804-807.
- Holland PM, Abramson RD, Watson R, Gelfand DH. Detection of specific polymerase chain reaction product by utilizing the 5'----3' exonuclease activity of *Thermus aquaticus* DNA polymerase. *Proc Natl Acad Sci U S A*. 1991 Aug 15;88(16):7276-80.
- Hirsch FR and Bunn PA. EGFR testing in lung cancer is ready for prime time. *The Lancet Oncology* 2009; 10(5), 432-433
- Hirsch FR, Varella-Garcia M, Bunn PA Jr, Franklin WA, Dziadziuszko R, Thatcher N, Chang A, Parikh P, Pereira JR, Ciuleanu T, von Pawel J, Watkins C, Flannery A, Ellison G, Donald E, Knight L, Parums D, Botwood N, Holloway B. Molecular predictors of outcome with gefitinib in a phase III placebo-controlled study in advanced non-small-cell lung cancer. *J Clin Oncol*. 2006 Nov 1;24(31):5034-42.
- Bell DW, Lynch TJ, Hasserlat SM, Harris PL, Okimoto RA, Brannigan BW, Sgroi DC, Muir B, Riemenschneider MJ, Iacona RB, Krebs AD, Johnson DH, Giaccone G, Herbst RS, Manegold C, Fukuoka M, Kris MG, Baselga J, Ochs JS, Haber DA.
- Epidermal growth factor receptor mutations and gene amplification in non-small-cell lung cancer: molecular analysis of the IDEAL/INTACT gefitinib trials. *J Clin Oncol*. 2005 Nov 1;23(31):8081-92.